

Foxp3 Transcription-Factor-Dependent and -Independent Regulation of the Regulatory T Cell Transcriptional Signature

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SUMMARY

The CD4⁺CD25⁺ lineage of regulatory T (Treg) cells plays a key role in controlling immune and autoimmune responses and is characterized by a unique transcriptional signature. The transcription factor Foxp3 had been thought to determine the Treg cell lineage, a hypothesis challenged by recent observations. We have performed a cross-sectional analysis of the Treg cell signature in Treg-like cells generated under a number of conditions, with or without Foxp3, to delineate the elements that can be ascribed to T cell activation, interleukin-2, transforming growth factor- β (TGF- β) signaling, or Foxp3 itself. These influences synergized to determine many of the signature's components. Much of the Treg cell signature was not ascribable to Foxp3 because it contained gene clusters that are coregulated with, but not transactivated by, Foxp3. Thus, a higher level of regulation upstream of Foxp3 determines the lineage, distinct from elements downstream of Foxp3 that are essential for its regulatory properties.

INTRODUCTION

CD4⁺ regulatory T (Treg) cells are central to the maintenance of immunological tolerance (Sakaguchi et al., 2006). This subset was initially identified as CD25⁺CD4⁺ T cells capable of controlling autoimmunity provoked by neonatal thymectomy or lymphopenia-induced dysregulation (Sakaguchi et al., 1995). Later, such cells were found to express the forkhead-winged helix transcription factor, Foxp3 (Khattry et al., 2003; Fontenot et al., 2003; Hori et al., 2003). Deficiencies in Foxp3 underlie the devastating lymphoproliferation and multiorgan autoimmunity in *Scurfy* mutant mice and human patients with the immunodysre-

gulation, polyendocrinopathy, and enteropathy, X linked (IPEX) syndrome (Ziegler, 2006).

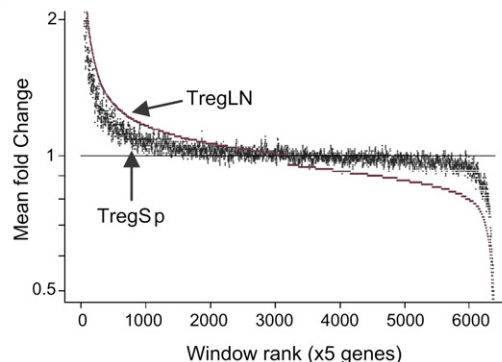
In the hematopoietic system, as elsewhere, differentiation into a particular lineage is thought to be determined by one or a combination of lineage-specification factors (Aliahmad and Kaye, 2006). In many instances, these lineage-committing factors integrate with the pre-established genomic terrain to produce a particular cellular phenotype or function (Souabni et al., 2002; Sun et al., 2005; He et al., 2005; Laiosa et al., 2006). In other cases, a factor (for example C/EBP α and β) can appear to be necessary and sufficient for determining a lineage (Xie et al., 2004; Laiosa et al., 2006). It has often been stated that Foxp3 acts as the "master regulator" or "lineage-specification factor" for Treg cells (Fontenot et al., 2005), because of the profound consequences of its absence and because ectopic expression of Foxp3 in CD4⁺ T cells generates a population with functional and phenotypic similarities to natural Treg cells (Hori et al., 2003). In contrast, several studies have hinted that Foxp3 might not have such a central role in determining the Treg cell lineage. A genomic analysis of Foxp3-transduced cells suggested that some elements of the Treg signature might be independent of this transcription factor (Sugimoto et al., 2006). More directly, cells with some, but not all, of the features of the Treg cell lineage are found in mice wherein the function of Foxp3 was inactivated but transcriptional activity at the locus remained detectable (Gavin et al., 2007; Lin et al., 2007).

A distinct gene-expression signature characterizes Foxp3⁺ Treg cells (Fontenot et al., 2005; Huehn et al., 2004; Herman et al., 2004). It includes not only certain cell-surface receptors long recognized as hallmarks of the lineage but also a wider array of transcription factors and other intracellular proteins. To elucidate the origin and determinism of this signature, and to clarify the importance of Foxp3 in specifying the Treg lineage, we have performed a cross-sectional analysis of the Treg transcriptional signature by combining gene-expression profiles from Treg cells obtained under many different conditions, by employing various inducers, and in the presence or absence of Foxp3. We have addressed the transcriptional impact of T cell activation through T cell

A

	Group	Cell type	Origin	Description	Treatment	TGF	Background
1	TregLN	CD4 Treg	LN	ex vivo LN		-	B6
2	TconvLN	CD4 Tconv	LN	ex vivo LN		-	B6
3	TregSp	CD4 Treg	spl	ex vivo spl		-	NOD&B6g7
4	TconvSp	CD4 Tconv	spl	ex vivo spl		-	NOD&B6g7
5	TregThy	CD4 Treg	thymus	ex vivo thymus		-	BDC/N
6	TconvThy	CD4 Tconv	thymus	ex vivo thymus		-	BDC/N
7	ActCD4AND	CD4 Tconv	spl	ex vivo AND TCRtg	activated <i>in vivo</i>	-	B6
8	CD4AND	CD4 Tconv	spl	ex vivo AND TCRtg	not activated	-	B6
9	ActCD4	CD4 Tconv	spl		activated <i>in vitro</i>	-	BDC/N
10	ActCD4TGF	CD4 Tconv	spl		activated <i>in vitro</i>	TGF	BDC/N
11	ActTreg	CD4 Treg	spl		activated <i>in vitro</i>	-	BDC/N
12	ActTregTGF	CD4 Treg	spl		activated <i>in vitro</i>	TGF	BDC/N
13	ActCD8	CD8 Tconv	spl	CD8	activated <i>in vitro</i>	-	BDC/N
14	ActCD8TGF	CD8 Tconv	spl	CD8	activated <i>in vitro</i>	TGF	BDC/N
15	sfActCD4	CD4 Tconv	spl	Foxp3 ^{0/0}	activated <i>in vitro</i>	-	BDC/N
16	sfActCD4TGF	CD4 Tconv	spl	Foxp3 ^{0/0}	activated <i>in vitro</i>	TGF	BDC/N
17	WTActCD4	CD4 Tconv	spl	Foxp3 ^{0/+}	activated <i>in vitro</i>	-	BDC/N
18	WTActCD4TGF	CD4 Tconv	spl	Foxp3 ^{0/+}	activated <i>in vitro</i>	TGF	BDC/N
19	FOXP3rv	CD4 Tconv	spl	retrovirus FOXP3	activated <i>in vitro</i>	-	BDC/N
20	CTRLrv	CD4 Tconv	spl	retrovirus Th1.1	activated <i>in vitro</i>	-	BDC/N
21	Foxp3rv	CD4 Tconv	spl	retrovirus Foxp3	activated <i>in vitro</i>	-	BDC/N
22	Ctrlrv	CD4 Tconv	spl	retrovirus GFP	activated <i>in vitro</i>	-	BDC/N

B



C

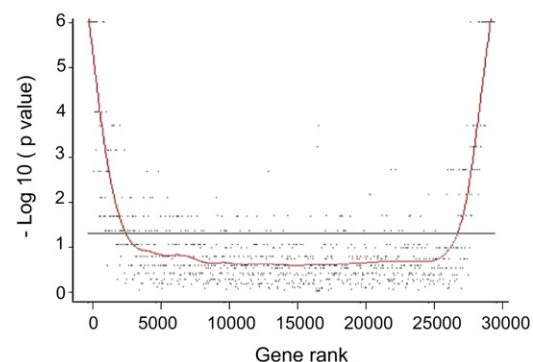


Figure 1. Populations of T Cells Used for Defining the Common Treg Cell Signature

(A) Summary of sorted populations used for analysis (the group names are used throughout).

(B and C) Depth of the Treg cell signature, estimated by ranked juxtaposition of independent Treg versus Tconv comparisons. (B) shows the mean fold change in the spleen Treg to Tconv comparison (black dots), in sliding windows ranked by fold change in the lymph node comparison (red dots). (C) shows the significance of the fold-change bias in the indicator comparison for the same sliding windows; the $p = 0.05$ threshold for significance is shown as a horizontal line.

receptor (TCR), interleukin-2 (IL-2), and transforming growth factor- β (TGF- β) signaling, as well as the influence of ectopic Foxp3 expression in conventional T cells. The combined data allowed the delineation of core-regulated gene clusters within the Treg cell signature, thereby providing a new perspective on the determinism of the lineage, the interplay between Foxp3 and TGF- β or IL-2, and the notion that a higher level of regulation actually determines the Treg cell lineage.

RESULTS

Defining the Treg Cell Signature

To define the components and origin of the Treg cell signature, we performed a meta-analysis that combined gene-expression profiles, generated in several parallel

experiments, from Treg cells or other Foxp3⁺ cells. As will be discussed below, the datasets listed in Figure 1A reflected a set of focused comparisons (e.g., the impact of TGF- β or the comparison of thymic and peripheral Treg cells), but their joint analysis also allowed a broader perspective on the Treg cell signature and its origin. In addition, we imported several relevant published datasets: comparative profiles from Treg and conventional T (Tconv) cells performed elsewhere (Fontenot et al., 2005; Gavin et al., 2007; Lin et al., 2007), a list of IL-2-responsive genes (Verdeil et al., 2006), and lists of Foxp3-binding genomic loci generated by chromatin immunoprecipitation (Zheng et al., 2007; Marson et al., 2007).

All gene-expression profiles were obtained from highly purified T cell populations sorted by flow cytometry. Populations used for *in vitro* activation were derived from naive

CD62L^{hi} Tconv or Treg cells so that we could avoid extraneous influences resulting from prior cell activation. To reduce variability, we pooled cells from multiple mice for sorting, and three replicates were generated for all groups. RNA from 0.5 to 2.5×10^5 cells was amplified, labeled, and hybridized to Affymetrix M430v2 microarrays that cover the quasi-totality of known murine genes. Raw data were preprocessed with the RMA algorithm in Gene-Pattern (Reich et al., 2006), and averaged expression values were used for analysis (full datasets deposited at NCBI under accession #GSE7460).

We first sought to obtain a comprehensive perspective on the Treg cell transcriptional signature. It has been difficult to decipher the true extent of differential gene expression in pairwise comparisons (Allison et al., 2006). Arbitrary cutoffs are often set on fold change or some statistical metric corresponding to false-discovery rates (FDRs) (Smyth, 2004) subjectively deemed acceptable; yet such strategies leave out a substantial fraction of the true but subtle differences that fail to meet this threshold. To circumvent this difficulty, we used a computational approach that tests for significance by determining whether differences observed in one experimental pair reproduce across independent comparisons that involve the same variable (here, the Treg versus Tconv cell difference; see [Experimental Procedures](#) in the [Supplemental Data](#) available online for detailed description). Here, we used three independently generated pairs of Treg-Tconv datasets (one data pair from the lymph node and two independent pairs from spleen; [Figure 1A](#), groups 1–4, each group in triplicate). As expected, specific changes were found on both ends of the spectrum, with transcripts both overrepresented and underrepresented in Treg cells compared to Tconv cells ([Figure 1B](#)). According to this analysis, the full range of changes in Treg cells involved a larger number of genes than previously recognized: Marked overexpression in Treg cells was detected for up to 1583 genes down to subtle fold changes of 1.13 (or 1804 genes deep for underexpressed genes at a fold change of 0.87 or less) ([Figure 1C](#)). Thus, the “identity” of Treg cells involves very subtle changes in regulatory programs for a wide number of genes.

In the analyses reported below, it was not possible to track the full extent of the transcriptional characteristics of Treg cells because this would involve changes at high FDR in any single comparison. For a robust definition of the transcriptional signature of mature Treg cells, we brought together results from several independent experiments: three profiles of lymph node and spleen Treg cells obtained here and one profile from an independent laboratory (Fontenot et al., 2005). We compiled the consensus peripheral Treg cell signature by calculating the Treg to Tconv fold change (Fc) ratios in the four analyses and retaining only those genes that showed a consistent 1.5-fold overexpression or underexpression in Treg cells in all four datasets (603 genes overall; 407 and 196 overexpressed or underexpressed in Treg cells, respectively; identities, values, and cluster for this gene are listed in [Table S1](#)).

Acquisition of the Treg Cell Signature

The Treg cell signature is defined, here and in other studies, on the basis of comparative gene-expression profiles from mature Treg cells isolated from peripheral lymphoid organs, such as the spleen or lymph node. It was of interest to know how much of the Treg cell profile is acquired early in the life history of these cells versus what might result from further maturation or other peripheral influences. Thus, we performed parallel comparisons of Treg cells and their Tconv counterparts isolated from the thymus, spleen, or lymph node. Highlighting in color the transcripts of the peripheral Treg cell signature defined above (overexpressed and underexpressed genes, red and blue, respectively) over the comparison of thymic Treg versus Tconv cells ([Figure 2A](#)) revealed that the vast majority of the signature's differential elements were already present in the thymus. This conclusion was confirmed in the “volcano plot” (fold change versus t test p value) of [Figure 2B](#), which further emphasized the skewing of genes of the common Treg cell signature: In total, 395/407 of the transcripts overrepresented in mature Treg cells were also overrepresented in thymic Treg cells ($\chi^2 p < 10^{-50}$); 185/196 were underrepresented. The differences found in comparisons of thymic and peripheral Treg cells were rather generic, in that they were evident also in comparisons of thymic and peripheral conventional CD4⁺ T cells ([Figures S1A](#) and [S1B](#)). Note that a simple Venn diagram comparison, analogous to that performed by Zheng et al. (2007), shows an overlap of 79% between genes up-regulated in thymic and peripheral Treg cells relative to their Tconv counterparts (data not shown). Thus, most of the Treg cell signature is already specified at an early stage of lineage commitment, independent of peripheral influences.

A Component of the Treg Cell Signature Is Related to Activation

Many of the cell-surface molecules, prototypical examples being CD25 (*Il2ra*), CTLA-4 (*Ctla4*), and GITR (*Tnfrsf18*) (Sakaguchi et al., 2006), expressed by peripheral Treg cells are also influenced by activation, a characteristic that hampered convincing identification of the Treg cell population for some time. This overlay might be due in part to the capacity of Treg cells to actively cycle in vivo (Fisson et al., 2003) and/or to the inherent self-reactivity of their TCR (Hsieh et al., 2004). To determine the contribution of mere activation to the Treg cell phenotype, we compared the signature of mature Treg cells with the changes elicited during T cell activation. The transcriptional changes occurring during T cell activation encompass those directly dependent on TCR (and costimulatory molecule) engagement, as well as those elicited secondarily by growth factors such as IL-2. To best encompass both types of signals, we generated datasets from two types of activated CD4⁺ T cells; first, we used CD4⁺ T cells activated in vivo by cognate antigen (cells from an AND TCR transgenic mouse, taken 60 hr after transfer into hosts that express the moth cytochrome c peptide recognized by the AND receptor [Obst et al., 2007]); second, to

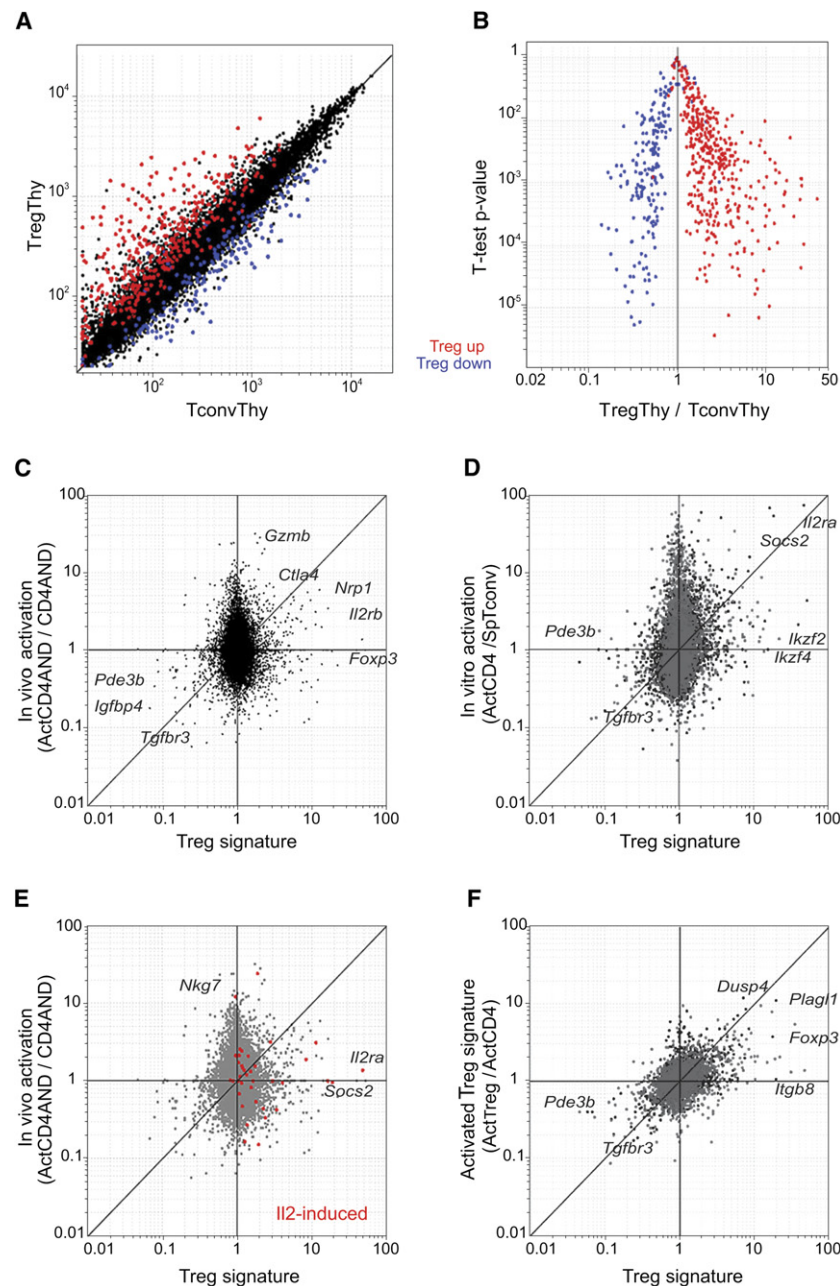


Figure 2. The Treg Cell Signature Is Imparted Early during Ontogeny and Contains Components of the Tconv Activation Response

(A) Comparison of expression values in Treg versus Tconv thymocytes, with the common signature of mature Treg cells highlighted in red (upregulated transcripts) and blue (downregulated transcripts).
 (B) "Volcano plot" representation (fold change versus t test p value) between Treg and Tconv cell expression profiles, restricted to the genes of the common Treg cell signature.
 (C) Fold change versus fold change (FcF) plot comparing the effect of in vivo activation of CD4⁺ T cells with the Treg cell signature (ratio of spleen Treg to Tconv).
 (D) As in (C), but the y axis represents changes elicited in naive CD4⁺ T cells activated in vitro in the presence of IL-2.
 (E) Same comparison as in (C), with IL-2-responsive genes defined from the data of Verdeil et al. (2006) highlighted in red.
 (F) FcF plot comparing the Treg signature before and after in vitro activation.

ensure maximum activation and full IL-2 signaling, we used CD4⁺ T cells activated in vitro with beads coupled with anti-CD3 plus anti-CD28, supplemented with IL-2. For the most part, the many changes elicited by activation

of CD4⁺ T cells bore no relationship to the Treg cell signature, as reflected by the predominant orthogonal streaks in Figures 2C and 2D. However, a distinct component of the transcripts responsive to either or both modes of

activation did belong to the Treg signature (falling on the diagonal quadrants in [Figures 2C and 2D](#); 232/407 and 136/196 of the overexpressed or underexpressed Treg signature genes, at a 1.5-fold cutoff for activation-induced changes). The Treg cell signature genes that overlapped with an activation response represented a distinct subset of the full activation response, primarily related to signal transduction rather than cell cycle, as shown by gene-ontology analysis (3', 5' cyclic-nucleotide phosphodiesterase, Rab GTPase binding, and transmembrane receptor protein serine-threonine kinase activity; χ^2 $p < 0.01$). Thus, the Treg cell signature does include a large component related to T cell activation, but this component is a selective subset of the full response normally elicited by T cell activation.

To confirm the influence of IL-2, we superimposed on the same comparison of changes a list of IL-2-induced genes, generated by Verdeil and colleagues in an analysis of IL-2 effects in synergy with TCR engagement ([Verdeil et al., 2006](#)) ([Figure 2E](#)). Essentially all of these IL-2-responsive genes showed a biased representation, by being overexpressed to some extent in Treg cells compared with Tconv cells activated in vivo.

Conversely, it was of interest to determine to what extent the distinctive transcriptional signature of Treg cells would be affected by full activation. Naive Treg and Tconv CD4⁺ cells were activated in vitro by anti-CD3 and anti-CD28 beads with IL-2, and the Treg-Tconv differential expression profile compared before and after activation ([Figure 2F](#)). Overall, the general distribution of genes along the diagonal indicated that much of the Treg cell signature persisted after TCR-mediated activation. However, close to 30% of the common Treg cell signature was lost during activation; this includes many candidate inhibitory receptors and transcripts associated with anergy, such as *Klrg1*, *Itgb8*, *Cd200*, *Maf*, *Folr4* (Folate receptor 4), and *Egr2*. Thus, certain elements of the Treg cell signature are related to T cell activation, whereas other elements of the signature are lost during Treg cell activation.

TGF- β Influences a Discrete Subset of the Treg Cell Signature

TGF- β signaling plays a role in maintaining Treg cell homeostasis, particularly in the periphery ([Li et al., 2006](#)). In addition, this growth factor can induce Foxp3 expression and might confer suppressive properties on CD4⁺ Tconv cells ([Chen et al., 2003a](#); [Fantini et al., 2004](#); [Wan and Flavell, 2005](#)). One might expect, then, that the TGF- β signaling pathway somehow contributes to the Treg cell signature. In order to parse out such a contribution, we generated gene-expression datasets from naive CD25⁻CD4⁺ Tconv cells cultured with anti-CD3 anti-CD28 beads in the presence of TGF- β and IL-2. Intracellular staining showed that >90% of cells became Foxp3⁺ at comparable amounts with that of Treg cells ([Figure 3A](#)), beginning in the first 24 hr of culture (data not shown). The profiles obtained with or without TGF- β were compared with the common Treg cell signature. The fold-change comparison of [Figure 3B](#) revealed that

TGF- β treatment elicited a number of the Treg signature transcripts, but not all of them. Some of the canonical Treg genes were induced, such as *Foxp3* (as expected from the cytometry profiles), *Nrp1*, *Itgae*, and *Gpr83*. However, TGF- β did not enhance the expression of several other Treg cell transcripts, such as *Il2ra*, *Socs2*, *Tnfrsf18*, and *Ctla4*, despite the nearly uniform and high expression of Foxp3 in the stimulated cells. One might argue that TGF- β was having a negative influence on the transcription of these genes by Foxp3; however, this was not the case because Treg cells cultured in the same conditions still exhibited the vast majority of the Treg signature ([Figure 3C](#)). A large number of transcripts with no relation to the Treg signature were also uniquely regulated by TGF- β : At a fold-change cutoff of 1.5, 970 and 845 genes were induced or repressed by TGF- β , of which only 123 and 72 belong to the canonical Treg cell signature. Thus, TGF- β readily induced Foxp3 in cultured naive T cells but influenced only a fraction of the transcriptional profile normally associated with Treg cells. This disjunction prompted us to test whether these TGF- β -induced cells exhibited the usual suppressive properties of Treg cells. This was clearly not the case, as illustrated in [Figures 3D and 3E](#): Suppression in the classic in vitro test ([Thornton and Shevach, 1998](#)) was very limited, with a partial effect visible only at high suppressor to target ratios ([Figure 3D](#)). This poor suppressive ability was observed in a number of experiments, in several different culture conditions and at different times of culture, with bead or plate-coated anti-CD3 plus anti-CD28 or with peptide stimulation in the presence of splenic antigen-presenting cells (data not shown). In addition, TGF- β -induced Foxp3⁺ cells were unable to confer protection against diabetes in vivo upon cotransfer with diabetogenic effector T cells, again unlike true Treg cells ([Figure 3E](#)). Thus, although TGF- β signaling generates components of the Treg cell signature including Foxp3, the full repertoire of transcripts and functional characteristics are not observed.

Foxp3 Profoundly Affects the TGF- β Response in CD4⁺ T Cells

The fact that a fraction of the Treg cell signature could be mimicked by TGF- β led us to ask the converse question: To what extent does Foxp3 participate in the response to TGF- β in CD4⁺ T cells? Because TGF- β is a strong immunomodulator in its own right, one might hypothesize that Foxp3 makes some contribution to its effect in T cells. We explored this question by examining CD4⁺ T cells from the *Scurfy* mutant mice, which harbor a frameshift mutation in the Foxp3 gene ([Ziegler, 2006](#)). Foxp3^{sf} mice usually develop a dramatic lymphoproliferative autoimmune phenotype, but the presence of TCR transgenes such as those encoding the BDC2.5 TCR predominantly prevented it, particularly in young mice (data not shown). The use of 10-day-old mice in combination with the TCR transgenes minimized the potentially confounding effects of inflammation on the TGF- β response; indeed, flow-cytometric analysis prior to culture showed negligible background activation in CD4⁺ T cells from

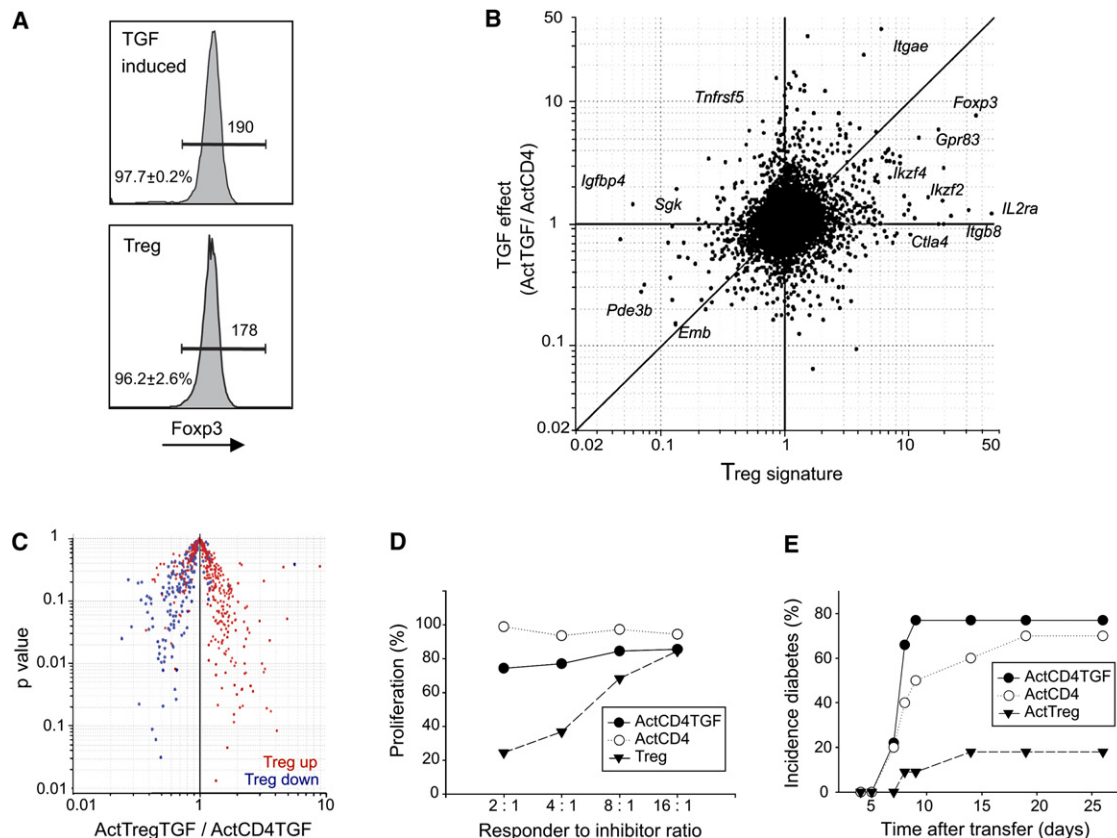


Figure 3. TGF- β Does Not Recapitulate the Entire Treg Cell Signature Despite Inducing Foxp3

(A) Foxp3 expression in TGF- β -treated cultures of naive CD4⁺ T cells, with cultured Treg cells for comparison (data representative of cytometry plots in postsort populations used for microarray analysis; the mean percentage of Foxp3⁺ ± SD shown for three independent experiments and representative mean fluorescence intensity [MFI]).

(B) FcF plot comparing changes induced by TGF- β in anti-CD3 and anti-CD28 + IL-2-activated T cells versus the Treg cell signature (Treg to Tconv ratio).

(C) Fold change versus p value plot of the effect of TGF- β for Treg cell signature genes.

(D) In vitro inhibition assay in which ex vivo-isolated Treg cells or in vitro-activated Tconv cells cultured in the presence or absence of TGF- β were tested as inhibitors of anti-CD3 activation of naive cells. Representative results of at least three independent experiments are shown.

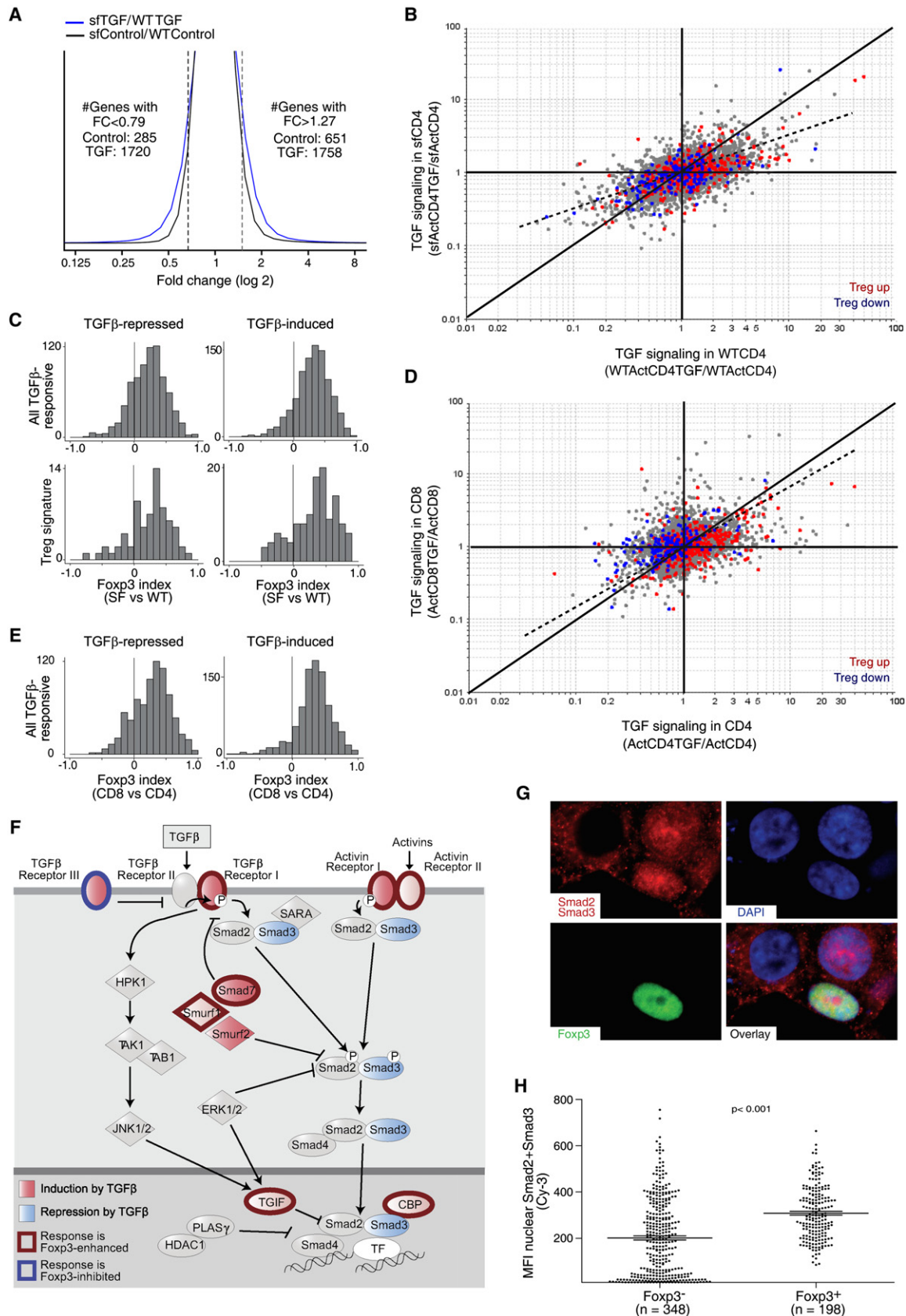
(E) Splenocytes from BDC2.5/NOD mice were activated in vitro under Th1-polarizing conditions (effector population) and transferred to NOD neonatal recipients with ActCD4 (n = 10), ActCD4TGF (n = 9), or ActTreg (n = 11) BDC2.5 T cells as inhibitor populations. Effector and inhibitor populations were transferred at a ratio of 2:1 respectively, and recipient mice were monitored for diabetes.

Foxp3^{sf} and wild-type littermates (Chen et al., 2005). As above, cells were stimulated with anti-CD3 anti-CD28 beads together with IL-2, in the presence or absence of TGF- β , and gene-expression profiling was performed. As expected, there was little divergence in the profile of Foxp3^{sf} and wild-type T cells from the control cultures, but a clear difference was revealed after treatment with TGF- β (Figure 4A).

By comparison of the TGF- β responses in CD4⁺ T cells from wild-type and Foxp3^{sf} mice, two important observations can be made (Figure 4B). First, the large number of changes elicited by TGF- β in wild-type CD4⁺ T cells remained predominantly present in Foxp3-deficient cells, as evidenced by the probes falling within the diagonal quadrants (Figure 4B). These include changes in a number of transcripts that belong to the common Treg cell signature (colored highlights), indicating that TGF- β is able to in-

duce these genes independently of Foxp3. Second, there was an attenuation of the overall response in Foxp3^{sf} cells, as indicated by the offdiagonal distribution of points: Induced genes were generally less induced, and repressed genes were less repressed (slope of the orthogonal linear regression = 0.47). Thus, although Foxp3 is not absolutely required for the bulk of gene regulation by TGF- β , its activity seems to augment the TGF- β response.

To better appreciate the impact of Foxp3 on TGF- β -responsive genes, we calculated a "Foxp3 index" that indicated, for each gene, the proportion of the TGF- β -induced responses in the absence of Foxp3 relative to the full responses seen in wild-type cells; an index of 0 denotes a TGF- β response independent of Foxp3; an index of 1 denotes a fully Foxp3-dependent response; and a negative Foxp3 index value indicates antagonism between TGF- β and Foxp3. This Foxp3 index corroborated our graphical



evidence that the TGF- β signature was augmented by Foxp3: most TGF- β -responsive genes showed a positive Foxp3 index, and this finding was true of genes either induced (mean = 0.30) or repressed (mean = 0.24) by TGF- β (Figure 4C, top panels). When the subset of TGF- β -responsive genes belonging to the Treg signature was considered, a similar distribution was observed (Figure 4C, bottom panels). A smaller proportion of transcripts had a negative Foxp3 index, indicating an antagonistic interaction between Foxp3 and the TGF- β signaling pathway. We attempted to trace the origin of these interactions by mapping the observed variations onto the TGF- β pathway (Derynck and Zhang, 2003). TGF- β elicits both positive and negative feedback on its signaling pathway: not only upregulation of the type I receptors but also downregulation of the key Smad3 transducer and induction of the inhibitory Smurf1, Smurf2, and Smad7. Foxp3 strongly enhanced several of these elements, in particular the induction of the TGF- β and activin type I receptors (Figure 4F, tabulated in Table S2). Foxp3 also repressed the TGF- β type III receptor, which inhibits signaling by TGF- β family members in concert with inhibins and β -arrestins (Lewis et al., 2000; Chen et al., 2003b). Thus, the net effect of Foxp3 might be to proximally increase sensitivity to TGF- β signals. The minority of genes showing antagonism between Foxp3 and the TGF- β response (negative Foxp3 index) might reflect a signaling branch that would be particularly sensitive to inhibition by Smad7 or Smurf1.

To verify these results in another cell type, we analyzed the response to TGF- β in CD8⁺ T cells, in which little to no Foxp3 expression was induced by TGF- β treatment (not shown), in keeping with previous observations (Kapp et al., 2006). The TGF- β -induced signature was present in CD8⁺ cells, yet muted relative to CD4⁺ T cells (Figure 4D), with a good concordance between the Foxp3 indices calculated by comparison of TGF- β responses of CD4⁺ T cells versus Foxp3^{sf} CD4⁺ cells versus CD8⁺ T cells (Figure 4E).

To corroborate the enhancing effect of Foxp3 on TGF- β -induced responses and begin to track its origin, we tested the effect of FOXP3 on the nuclear and cytoplasmic distribution of the Smad proteins, the principal mediators of TGF- β -dependent transcriptional activity. Smad2 and Smad3 continuously shuttle between the cytoplasm and nucleus at steady state but are retained in the nucleus,

and are hence transcriptionally active, after TGF- β -mediated phosphorylation. Smad2 and Smad3 nucleocytoplasmic shuttling can be regulated at multiple levels, including TGF- β receptor activity, interactions with nuclear binding partners, and nuclear phosphatase activity such as PPM1A (Hill, 2006) and is thought to be responsible for the duration and strength of TGF- β signaling. We transfected 293 cells with a vector containing human FOXP3 and compared by confocal microscopy the nuclear localization of Smad2 and Smad3 24 hr after TGF- β stimulation. FOXP3-positive cells showed a clear and significant bias in the distribution of nuclear Smad2 and Smad3 protein, relative to FOXP3-negative cells in the same cultures (Figures 4G and 4H; transfection with empty vector had no such effect—not shown). FOXP3 affected the proportion of cells with high nuclear Smad2 and Smad3, rather than the maximum level of nuclear accumulation; it also did not affect the distribution of Smad2 and Smad3 during acute TGF- β stimulation after 1 hr (data not shown), suggesting that FOXP3 might prolong nuclear retention rather than influx of Smad2 and 3. Thus, FOXP3 can directly influence TGF- β signaling, even in non-T cells.

Dependence of Treg Cell Signature on Foxp3

The surprising observation that TGF- β -treated cells exhibited full Foxp3 expression but only some elements of the Treg cell signature underlined the question of Foxp3's true contribution to the Treg cell signature. To assess this contribution most directly, we activated purified naive CD4⁺ T cells and transduced them with a retroviral vector encoding FOXP3 and a Thy1.1 reporter. Parallel transductions were performed with vectors encoding human FOXP3 or mouse Foxp3, and these were used for independent corroboration. Reporter-positive cells were sorted from these populations for gene-expression profiling; as indicated, these cells were >95% Foxp3⁺, with a mean fluorescence intensity almost equivalent to Treg cells (Figure 5A).

Comparison of the profiles highlighted Foxp3's role as a transcriptional repressor as well as an activator, consistent with previous studies (Fontenot and Rudensky, 2005; Campbell and Ziegler, 2007). When the response to FOXP3 was compared with the Treg cell signature (Figure 5B), it was apparent that, here again, FOXP3

Figure 4. Scurfy CD4⁺ T Cells Show a Blunted Response to TGF- β Signaling

(A) Histogram analysis of cumulative fold-change differences between wild-type (WT) and scurfy (sf) CD4⁺ T cells.

(B) FcFc analysis of TGF- β signaling in WT and sf CD4⁺ T cells.

(C) Histogram analysis of the Foxp3 index (see Experimental Procedures) for TGF- β -responsive genes outside (top) and within (bottom) the Treg cell signature.

(D) FcFc analysis of TGF- β signaling in CD8⁺ and CD4⁺ T cells.

(E) Histogram analysis of the Foxp3 index for TGF- β -responsive genes in CD8⁺ and CD4⁺ T cells.

(F) Pathway analysis of TGF- β signaling color coded by expression-value changes found in WT or sf CD4⁺ T cells (derived from ingenuity pathways analysis, ingenuity systems).

(G) FOXP3 maintains nuclear Smad2 and Smad3 retention after TGF- β signaling. We transfected 293 cells with FOXP3 and cultured them for 24 hr in the presence of TGF- β to determine nuclear Smad2 and Smad3 localization. A representative image of 293 cells stained with anti-Smad2/3 (red), DAPI (blue), anti-Foxp3 (green) and the overlay of the three images are shown.

(H) Quantification of mean fluorescence intensity of nuclear Smad2 and Smad3 in Foxp3-positive or -negative cells within the same culture (as depicted in [G]). Representative data are shown for one of three independent experiments. Points represent nuclei that were masked individually by DAPI staining for determination of Cy-3 (anti-Smad2/3) MFI. Horizontal bars represent the mean \pm SEM; $p < 0.001$ by Mann-Whitney test.

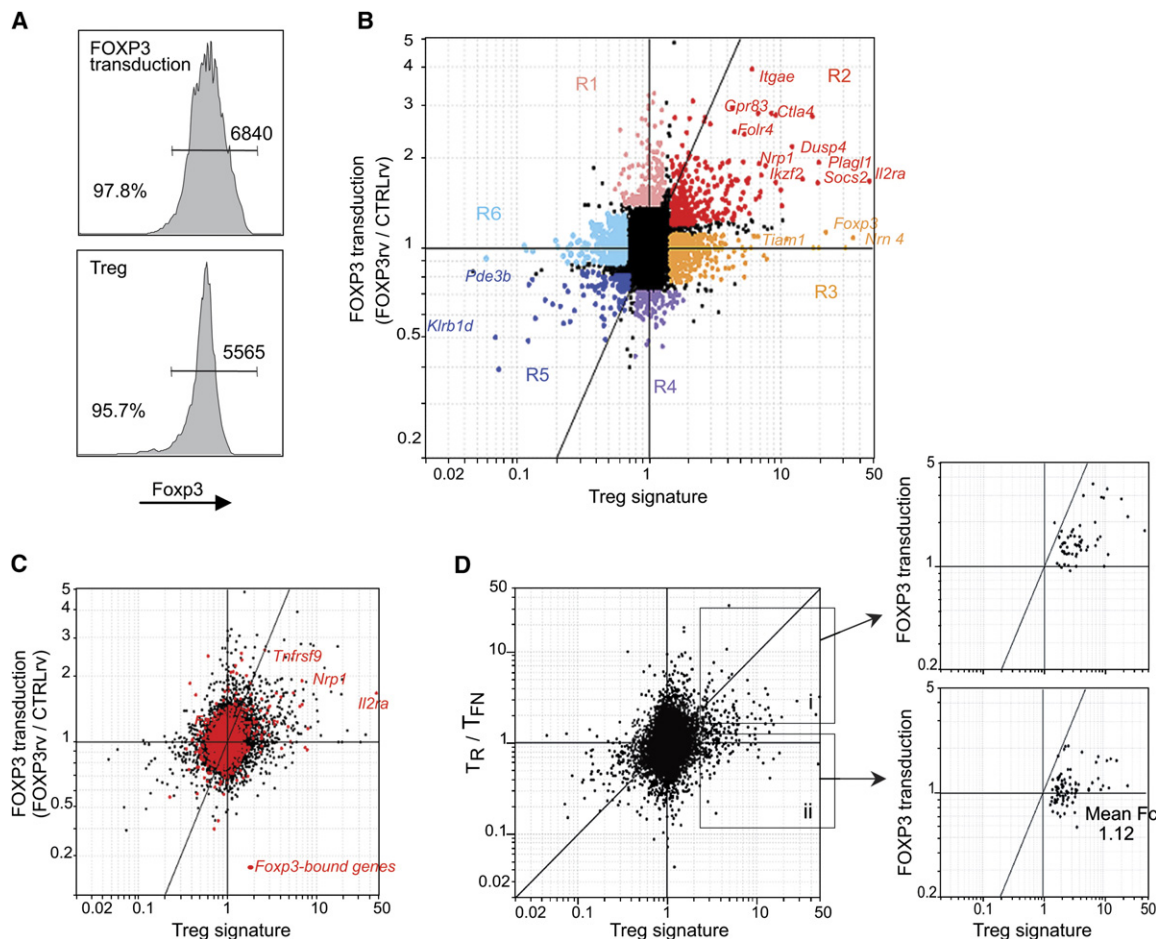


Figure 5. Retroviral Transduction of FOXP3 in Tconv Cells Regulates a Population of Genes within the Common Treg Cell Signature

(A) Foxp3 expression in FOXP3-transduced cultures of activated CD4⁺ T cells, with Treg cells for comparison.

(B) FcFc plot comparing the effect of FOXP3 transduction with the Treg cell signature. Probes and expression values for genes in the R1–R6 regions are found in Figures S3–S8.

(C) FcFc plot as in (B), highlighting genes whose promoter elements are bound by Foxp3 (Zheng et al., 2007; Marson et al., 2007).

(D) FcFc plot comparing the difference in gene expression between Foxp3⁺ Treg cells and Foxp3null Treg cells (T_R/T_{FN} data from Gavin et al. [2007] or Lin et al. [2007]) with the TregSP profile. Gate “i” identifies genes whose overexpression in Treg cells requires functional Foxp3, whereas those in gate “ii” remain overexpressed in Treg cells in the absence of functional Foxp3 (in practice, gates were defined by the overlap between data from Gavin et al. [2007] and Lin et al. [2007], to limit the effect of noise in these datasets); the panels at the right display the genes in gates “i” and “ii,” on the FOXP3 transduction/Treg cell signature FcFc plot.

transduction reproduced only a fraction of the Treg cell signature. Of the transcripts overexpressed in Treg cells, some were clearly induced by FOXP3 (188 of 407 probes representing the consensus Treg “up” signature, even with a very unrestrictive cutoff of 1.2-fold; region 2 of Figure 5B), but 180 were unaffected or even repressed (region 3). For genes underexpressed in Treg cells, the direct effect of FOXP3 was also limited (54 of 196 probes from the Treg “down” signature were repressed by FOXP3 transduction, versus 108 unaffected or even slightly induced; regions 5 and 6). The set of transcripts directly affected by FOXP3 included several genes considered hallmarks of Treg cells; these genes include *Il2ra*, *Ctla4*, *Tnfrsf18*, *Itgae*, *Gpr83*, and *Nrp1*. Treg cell transcripts that did not respond to FOXP3 included *Foxp3* itself (the array detects the endogenous *Foxp3* transcripts, not the

transduced human mRNA), along with *Nrn1* and *Itgb8*. Conversely, FOXP3 transduction elicited changes in a number of transcripts that do not belong to the Treg signature (regions 1 and 4); these changes were reproducible, in that many were also elicited by transduction of mouse *Foxp3* (not shown). Hence, Foxp3 alone is unable to elicit the full range of transcriptional features of the Treg signature; in addition, it induces independent changes that do not belong to the Treg cell signature.

Recent studies from two independent groups have used chromatin immunoprecipitation to identify regions of genomic DNA that associate with Foxp3 in vivo (Zheng et al., 2007; Marson et al., 2007). We thought it of interest to compare these data with the transcriptional signature derived here. To that end, we compiled a robust list of the Foxp3-binding genes by applying the statistical

cutoffs proposed by both studies and selecting 2192 genes found in at least two conditions (combining arbitrary cutoffs is certain to overlook some true positives, but should yield a list of true Foxp3-binding loci with very few false positives). These Foxp3-binding genes are highlighted on the plot of Figure 5C. As noted (Zheng et al., 2007; Marson et al., 2007), many of these loci are not transcriptionally responsive to FOXP3, nor do they belong to the Treg cell signature (those at the center of Figure 5C), but there was also an overrepresentation of transcripts influenced by FOXP3 and/or belonging to the Treg signature ($\chi^2 p = 2 \times 10^{-9}$ and 8×10^{-3} , respectively). In addition, several Foxp3-binding loci did coincide with transcripts affected by FOXP3 transduction but did not differ in Treg versus Tconv cells (corresponding to region 1 or region 6 of Figure 6B). Overall, the Foxp3 binding information supported the notion that other factor(s) might complement and modify, by enhancing or suppressing, the activity of Foxp3 in generating the overall Treg cell signature.

The inability of Foxp3 to influence the expression of all members of the Treg cell signature suggests that some of the characteristic profile is elicited by factors other than Foxp3. To corroborate these results, we utilized previously described datasets that were from the Rudensky and Chatila laboratories and that compared transcripts from normal Treg cells with those from a population of Foxp3-negative Treg “look-alikes” (T_{FN}) that appeared in mice in which the Foxp3 coding region was replaced by a GFP reporter (Gavin et al., 2007; Lin et al., 2007). As illustrated in Figure 5D (left panel), the expression of a number of transcripts characteristic of the Treg cell signature was not affected by the loss of Foxp3 in T_{FN} cells. Conversely, only a subset of those genes that were affected by the loss of Foxp3 in the genetic-variant context (T_{FN}) responded to Foxp3 transduction (Figure 6D, right panel). Thus, overlapping Foxp3-independent components of the Treg cell signature can be demonstrated in two independent experimental contexts.

Coregulation

To further substantiate the Foxp3 independence of certain elements of the Treg cell signature, we made use of the complete array of datasets and searched for correlations between the expression of Foxp3 and other Treg cell signature genes. Our rationale was that the expression of genes directly activated by Foxp3 might be expected to tightly correlate with that of Foxp3 itself across the datasets. This was clearly the case for some transcripts, whether positively or negatively associated with Foxp3 transcript levels (e.g., *Hipk2*, *Pde3b*; Figure 6A), but not for all: The expression of *Ctla4* correlated only very loosely with that of Foxp3 (however, *Ctla4* does correlate with other Treg signature genes such as *Swa70*). This dichotomy is generalized in the histogram of Figure 6B, which displays the Pearson correlation coefficient for all genes of the Treg cell signature relative to Foxp3. Approximately one-third of the transcripts correlated with Foxp3 (coefficient >0.5), whereas at the other end of the spectrum, some genes exhibited the strong negative correlation expected

for those repressed by Foxp3 (coefficient <-0.5). In between, a large number of Treg cell signature genes showed little or no correlation with Foxp3, suggesting an independent or at least a more complex mode of regulation.

We then determined whether other strongly correlated clusters might be found within the Treg signature by constructing a complete matrix of gene-gene correlations across all datasets and using a partition clustering algorithm to group these genes into discrete clusters (Figure 6C, cluster listings found in Table S1). These clusters did show distinct expression characteristics: cluster 1, which tightly correlated with Foxp3 expression, included a number of genes responsive to TGF- β or FOXP3 transduction. In contrast, clusters 4 or 5 were more directly responsive to TCR and IL-2 cues and relied little on the presence of Foxp3. Cluster 6 was also of particular interest because many of these signature genes were not influenced by activation, TGF- β signaling, or FOXP3 transduction. Thus, the Treg cell signature contains clusters of coexpressed genes whose regulation does not appear directly connected to Foxp3.

Close inspection of the expression profiles of Figure 6C revealed that, even for the genes in clusters most tightly correlated with Foxp3, its transduction did not uniformly lead to transactivation. We examined this relationship further by plotting, for each gene, the correlation with Foxp3 relative to the impact of FOXP3 transduction (Figure 6D). Several distinct groups of genes emerged. Those of group 1 were clearly transactivated by FOXP3, yet they correlated poorly across the grouped datasets, most probably because they are influenced by other inducers such as IL-2 or TGF- β (*Il2ra*, *Socs2*, and *Itgae*). The transcripts of groups 2 and 3 all correlated well with Foxp3. Those of group 2 were also transactivated by Foxp3, suggesting that they come under Foxp3 control, direct or indirect. In contrast, although Foxp3 correlated well with the genes of group 3, it was unable to induce their expression (probes from groups 1–3 are found in Tables S9–S11). This pattern of expression is what one would expect of genes controlled by a higher-order transactivator that would also control Foxp3. These proposed relationships are illustrated in Figure 6E.

This study promotes the concept that a number of influences conspire to generate the Treg cell signature. Taking the 603 genes of the by now well-defined consensus Treg cell signature, our findings show that it is possible to distinguish those that are influenced individually by Foxp3, TGF- β , or activation through TCR and IL-2R triggers. As shown in Figure 6F, genes affected by IL-2 and/or TCR activation constituted the majority of the Treg cell signature, but many of these genes could also be influenced by TGF- β or Foxp3. Conversely, the majority of genes responsive to Foxp3 were also responsive to another inducer, either IL-2 or TGF- β . Thus, the characteristic Treg cell signature results from a complex interplay, rather than from any single influence. To some extent, this interplay is wired into the reciprocal influences that each of these inducers has on the other's signaling pathways: Foxp3 and IL-2 both induce CD25, the α chain of the

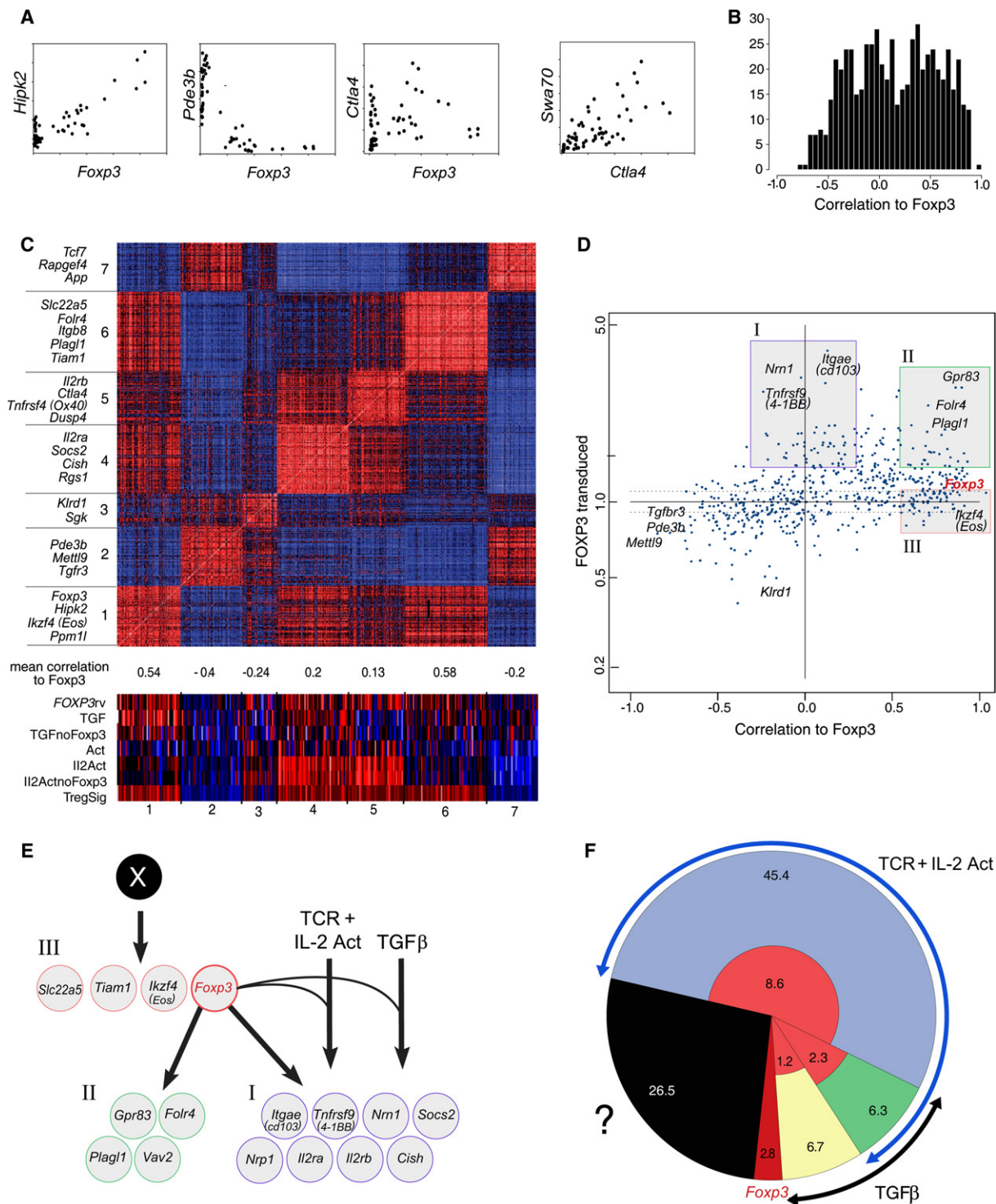


Figure 6. Clusters of Coregulated Genes within the Common Treg Cell Signature

(A) Expression value plots of *Foxp3* versus other genes in the Treg cell signature; each dot represents an independent dataset, as listed in Figure 1A. (B) Histogram representation of the correlation between the expression of *Foxp3* and all other genes within the common Treg cell signature. (C) Cluster analysis of expression correlations between all genes within the common Treg cell signature, clustered by a partition clustering algorithm ($k = 7$). Red represents positive correlations, blue represents negative correlations, and black represents no correlation. Representative genes within each cluster are identified to the left of the upper panel with the corresponding cluster number, see Table S1 for a full listing of genes inside the clusters. The bottom portion shows a heatmap representation (for the Treg cell signature genes aligned according to their cluster membership as above) of the differences in expression induced by *FOXP3* transduction (*FOXP3*rv), TGF- β treatment in the presence or absence of *Foxp3* (TGF and

IL-2R, thus potentiating IL-2 signaling; perhaps more unexpectedly, Foxp3 broadly enhances TGF- β signaling.

DISCUSSION

These results compel one to revisit the notion of Foxp3 as a simple “master regulator” or “lineage-specification factor” for the Treg cell lineage. This transcriptional regulator had been assigned such a role on the basis of the severe autoimmune manifestations that appear in its absence and on its ability to confer some degree of suppressive activity upon retroviral transduction (Fontenot et al., 2003; Hori et al., 2003). The very concept of “lineage-specification factor” implies that such a factor be both necessary and sufficient for the existence and phenotypic characteristics of the lineage. Accumulating evidence, presented here and by others, has identified circumstances in which these two requisites are not met by Foxp3: Whether after induction by TGF- β or after retroviral transduction, Foxp3 is not sufficient to elicit the full Treg cell signature (similarly, activated human Tconv cells express some Foxp3, yet show no suppressive characteristics [Walker et al., 2003; Allan et al., 2007]). Conversely, the Rudensky and Chatila laboratories have shown that Foxp3 is not necessary because cells with several of the characteristics of the Treg cell lineage (including transcriptional activity at the Foxp3 locus) can be selected and persist *in vivo* in its absence (Gavin et al., 2007; Williams and Rudensky, 2007; Lin et al., 2007), consistent with the existence of Treg-like cells in some IPEX patients (Bacchetta et al., 2006). In hindsight, the very concept of a “lineage-specification factor” is likely to be an oversimplification. It derives from the “operon” concept of bacterial genetics (Jacob et al., 1960); in this concept, an entire train of genes and associated functions are placed under the control of a single regulatory element. But unique lineage-specification factors might not exist in mammalian systems; some factors that might have appeared at first to specify a unique lineage (such as Pax5 for B lymphocytes) were later shown to be only part of a more complex regulatory combination (Medina and Singh, 2005). Indeed, lineage specification in lymphoid systems might be as dependent on the absence of an inhibitory factor(s) as on the presence of an inducer (Souabni et al., 2002; Xie et al., 2004; Rothenberg and Dionne, 2002).

If not Foxp3, then what factor(s) impose the transcriptional characteristics of the Treg cell lineage? One might

have suggested that peripheral influences expand on a signature that is partially acquired in the thymus at the time of lineage specification; this is clearly not the case, because virtually all of the signature is already acquired in thymic Treg cells, implying that the factor(s) responsible for Treg cell generation rapidly induce a phenotype that is actually quite robust and because the profile remains clearly identifiable even after full TCR and IL-2 activation. Our studies with thymic Treg cells contradict other results suggesting that a substantially different transcriptional profile is evident in thymic versus peripheral Treg cells (Zheng et al., 2007; Gavin et al., 2007), but we believe that these apparent differences are most likely to be a result of technical differences with these previous data sets. The coregulation analysis, demonstrating the existence of a group of genes coregulated with Foxp3, but not induced directly by it, suggested the presence of a higher-order regulator, which would determine the expression of Foxp3 and of its coregulated genes. Note that this higher-order factor need not be a single element but just as well might be a combination of factors. In this model, Foxp3 would then serve to transactivate an additional set of genes but perhaps also to potentiate and reinforce the effect of the higher-order factor (as suggested by the Rudensky laboratory [Gavin et al., 2007]). There is no indication, however, that Foxp3 transactivates its own expression in a positive feed-forward loop, as proposed elsewhere (Gavin et al., 2007). The set of functions directly under Foxp3 control does include elements essential for Treg immunoregulatory activity, and such a finding explains why Foxp3 was initially thought to specify the entire lineage, rather than only a fraction of its effector characteristics.

There is clearly a complex relationship between TGF- β and Foxp3 (and Treg cells more generally). TGF- β is intimately associated with the survival and function of Treg cells (Li et al., 2006) and can induce Foxp3 as well as a sizable component of the Treg signature; some of this induction occurs independently of Foxp3 because it is also observed in TGF- β -treated CD8⁺, or Foxp3^{sf} CD4⁺, cells. Although TGF- β itself is not required for the specification of the Treg cell lineage in the thymus, one might speculate that signals from other members of the TGF family of cytokines (for instance through activins expressed in the thymus) might be required. Conversely, the present data showed that Foxp3 enhances the response to TGF- β , at least in part by modulating the expression of the type I

TGF α Foxp3), TCR and IL-2 activation (Act, ActIL2, and ActIL2noFoxp3), and the Treg cell signature (Treg to Tconv). Heatmaps have been row normalized. Numbers between the two panels indicate the average correlation to Foxp3 expression within each cluster.

(D) Scatter plot of the changes induced by FOXP3 retroviral transduction versus the correlation coefficient values to Foxp3 for genes of the common Treg cell signature. Box I identifies genes that can be induced by FOXP3 but are not correlated with Foxp3 expression across the dataset; box II identifies genes that are both induced by FOXP3 and show a strong correlation with Foxp3 expression. Box III contains genes that are strongly correlated with Foxp3 but are not induced by FOXP3. See Tables S9–S11 for gene identities and values.

(E) Schematic representation of the gene-expression hierarchies derived from data within (C) and (D).

(F) Summary of genes within the common Treg cell signature whose expression can be affected by TCR and IL-2 activation (blue quadrant; defined as >1.5-fold change in expression between wtActCD4 versus TconvSp) or TGF- β (yellow quadrant; defined as >1.5-fold change in expression between wtActCD4TGF versus TconvSp, but <1.5-fold change between wtActCD4 versus TconvSp) or both (green quadrant, defined as >1.5-fold change in expression between wtActCD4 versus TconvSp, and >1.5-fold change in expression between wtActCD4TGF and wtActCD4) independently of Foxp3. Those genes whose expression can be directly regulated by FOXP3 transduction are indicated in red. Foxp3 inducible genes whose expression can also be regulated by activation or TGF- β signaling are indicated in the red overlap of pie slices.

and type III receptors and by increasing the nuclear retention of Smad2 and Smad3. This retention could result from a direct FOXP3-Smad interaction, as has been described previously for FOXP3 (Randall et al., 2004) or indirectly from a FOXP3-induced function. Although this point remains to be determined, these results provide a mechanism for FOXP3's influence on TGF- β -induced transcription.

TGF- β -induced Foxp3⁺ cells had little or no suppressive ability in our hands, and their Foxp3⁺ status was unstable, as also seen by others (Floess et al., 2007). In contrast, other groups (e.g., Davidson et al. [2007]) have reported that TGF- β -induced Foxp3⁺ cells have full immunoregulatory capacity. We surmise that subtle technical differences explain these divergent results, and it will be highly informative to track the root of the differences because this information should help to identify key elements of Treg activity. Yet the key point here is that the high expression of Foxp3 present in these TGF- β -treated cells are unable to elicit the full Treg cell signature. Indeed, we have observed the same genomic "hole" when profiling TGF- β -converted Foxp3⁺ cells generated in a laboratory in which TGF- β -Tregs do have suppressive efficacy (data not shown; J. Hall and Y. Belkaid, personal communication).

In conclusion, our findings emphasize the need for a revised view of Foxp3's role in Treg cells. Foxp3's relatively minor contribution in sculpting the Treg cell transcriptional landscape challenges the current view of this transcription factor as a "master regulator" and suggests the involvement of additional elements in this process. Identifying this (these) factor(s) will be essential for understanding Treg cell specification and function and their role in the prevention of autoimmune disease.

EXPERIMENTAL PROCEDURES

Mice

NOD/LtDOJ, C57BL/6J, C57BL/6.H2g7, BDC2.5/NOD TCR tg mice (Katz et al., 1993) and BDC/N.Foxp3^{sf} mice (Chen et al., 2005) were bred in our SPF Joslin facility or purchased from Jackson Laboratories (protocol 99-20, approved by the Joslin Diabetes Center's International Animal Care and Use Committee).

Cells

Treg and Tconv cells were harvested from the lymph node (inguinal, cervical, and mesenteric from 32- to 36-week-old B6 mice), spleen (from 6-week-old B6.H2g7 or NOD mice) or thymus (from 4- to 6-week-old BDC/N mice) for ex vivo populations. In vivo activated and naive AND CD4⁺ T cells were isolated as described previously (Obst et al., 2007). Cells used for in vitro activation were obtained from pooled lymph node and spleen of 4- to 6-week-old BDC/N mice or from 10-day-old BDC/N.Foxp3⁺ and BDC/N.Foxp3^{sf} mice.

Cell Sorting and Flow Cytometry

For ex vivo populations, cells were sorted as B220⁺CD8⁺CD11b⁺CD4⁺ and either CD25^{hi} (Treg) or CD25⁺ (Tconv). Naive cells for in vitro activation were sorted as B220⁺CD8⁺CD11b⁺CD4⁺CD69⁺CD62L^{hi} and either CD25^{hi} (Treg) or CD25⁺ (Tconv). CD8 T cells for in vitro activation were sorted as B220⁺CD8⁺CD11b⁺CD4⁺CD69⁺CD62L^{hi}. Cells from 10-day-old BDC/N.Foxp3⁺ and BDC/N.Foxp3^{sf} were enriched by anti-CD4 MACS beads. After 4 days of in vitro culture, cells were sorted again before being processed for microarray analysis or used in func-

tional studies. We sorted ActCD4 and ActCD4TGF cells on CD25 positivity to ensure effective activation (top 45% for both populations). ActTreg and ActTregTGF were also sorted as CD25 expression (>95% CD25^{hi}). CD8 T cells were sorted as CD8^{hi}. Cells from wtActCD4, wtActCD4TGF, sfActCD4, and sfActCD4TGF were all sorted as CD4⁺ and CD25^{hi} (top 45%). Retrovirally transduced cells were sorted on expression of the vector's selectable marker (Thy1.1 or GFP). Postsort populations were assessed for Foxp3 expression by intracellular staining.

In Vitro Activation

T cells were activated with anti-CD3 and anti-CD28 coated beads (Dynal) at a concentration of one bead/cell in the presence of 20 U/ml recombinant human IL-2 (2000 U/ml for Treg cells, Proleukin, Chiron) with or without 25 ng/ml recombinant TGF- β (PeproTech) for 4 days.

Retroviral Transduction of FOXP3

293FT cells were transfected with retroviral expression plasmids (MSCV IRES-Thy1.1/GFP empty or encoding wild-type human or mouse Foxp3) with TransIT-293 (Mirus) according to the manufacturer's instructions. Tconv cells were activated in vitro with anti-CD3 and anti-CD28 coated beads in the presence of 20 U/ml IL-2, then spin-infected with retrovirus supernatant at 48 hr. Cells were then cultured for an additional 48 hr (total in vitro culture of 4 days).

In Vitro Inhibition Assay

A total of 1×10^5 BDC/N splenocytes (containing 2×10^4 CD4⁺ T cells) were used as responders. The indicated BDC/N T cell inhibitor populations (ActCD4, ActCD4TGF, or Treg ex vivo) were cocultured at various concentrations for 4 days in the presence of 1.5 μ g/ml anti-CD3 antibody ([³H] Thymidine pulse in last 18 hr). Results are averaged from triplicate and standardized to wells with no inhibitor population.

Induction of Diabetes in NOD Neonates

We generated effector cells from BDC/N splenocytes by culturing them in the presence of anti-CD3/CD28 beads, 20 U/ml IL-2, and 1 ng/ml IL-12 (PeproTech) for 4 days. The indicated inhibitor populations (ActCD4, ActCD4TGF, and ActTreg/ActTregTGF) were mixed with effectors at a 1:2 ratio (0.5×10^5 : 1×10^5 , inhibitor: effector) and transferred to neonatal NOD mice (1 to 3 day old).

Nuclear Localization of Smad2 and Smad3

293FT cells were transfected with retroviral expression vector containing human FOXP3 (as above). Cells were transferred to chambered coverglass slides (Nunc) 8–24 hr later, rested for 12 hr, and cultured with TGF- β for 24 hr. Cells were fixed and permeabilized for 2 hr; this was followed by staining with anti-Thy1.1 FITC, rabbit anti-Smad2/3 (Cell Signaling), then by staining with donkey anti-rabbit Cy-3 (Jackson), anti-Foxp3 APC, and DAPI. High-power images (63 \times) were acquired with a Marianas microscopy workstation with Slidebook software (3i, Denver).

Microarrays

RNA was prepared from sorted cell populations as described (Trizol [Yamagata et al., 2004]). RNA was amplified for two rounds (MessageAmp aRNA, Ambion), biotin labeled (BioArray High Yield RNA Transcription Labeling, Enzo), and purified with the RNeasy Mini Kit (QIAGEN). The resulting cRNAs were hybridized to M430 2.0 chips (Affymetrix). All cell populations identified in Figure 1A were generated in triplicate (except Foxp3rv and Ctrlrv, which were in duplicate). Raw data were normalized with the RMA algorithm implemented in the "Expression File Creator" module from the GenePattern software package (Reich et al., 2006) (www.broad.mit.edu/cancer/software/genePattern/). We filtered probes to exclude nonvariable low- and high-expression values by using the "Preprocess Dataset" module from GenePattern and reducing the data set to 29,947 probes. Data were visualized with the "Multiplot" and "SignatureDepth" modules from GenePattern (R.M., unpublished data); see Supplemental

Experimental Procedures). Gene-ontology Identifiers were curated from www.geneontology.org. For the cluster analysis of Figure 6, a matrix of correlation coefficients (603 × 603 probes) was calculated for all genes of the Treg signature, on the basis of their averaged expression values in the 22 different conditions; this matrix was used as input for the “clara” partition clustering algorithm in S-Plus.

Supplemental Data

Additional Experimental Procedures, one figure, and eleven tables are available at <http://www.immunity.com/cgi/content/full/27/5/786/DC1/>.

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